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MEDICAL
INTELLIGENCE
UNIT 24

Peptide-Based Cancer Vaccines

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CHAPTER 1

Identification and Selection of T-Cell Epitopes Derived from Tumor-Associated Antigens for the Development of Immunotherapy for Cancer

Esteban Celis

Because the immune system has the capacity to recognize and in many cases destroy tumor cells, significant efforts are being devoted to the development of immune-based therapies for cancer. Both cytotoxic T lymphocytes (CTL) and helper T lymphocytes (HTL) have been shown to react with antigens expressed by tumor cells and as a result, establish protective and therapeutic effects. Since CTL and HTL recognize antigens in the form of peptide complexes with major histocompatibility complex (MHC) surface molecules (HLA in humans), it is necessary to identify the nature of tumor-derived peptides that can elicit T-cell responses capable of inhibiting tumor-cell growth. The overall objective of our work is to identify peptides derived from sequences of several known tumor-associated antigens (TAA) that are capable of stimulating CTL and HTL against tumor cells. The amino acid sequences of TAA are screened for the presence of peptides containing MHC binding motifs. Corresponding peptides are then synthesized and tested for their capacity to elicit in vitro T-cell responses to tumor cells and corresponding TAA as a final proof that they truly represent T-cell epitopes. As a consequence of these studies, the identified tumor-reactive T-cell epitopes can be developed into therapeutic compounds to treat commonly found epithelial cancers (breast, gastrointestinal and lung). The remaining challenges are how to select the most appropriate mode of vaccination and how to evaluate the effectiveness of immunotherapy in the cancer setting.

Introduction

The incidence of many types of tumors including breast, prostatic, colorectal and lung carcinomas continues to rise in the majority of developed and underdeveloped countries. Most importantly, there is a desperate need to develop non-toxic therapies to eliminate disease, prevent tumor recurrences and inhibit metastatic dissemination, all which should prolong survival while maintaining a good quality of life. Immunotherapy must be considered as the best alternative to accomplish this goal. The purpose of this Chapter is to describe our group's approach to develop effective immune-based therapies for the treatment of commonly found types of cancer. Our belief is that T-lymphocytes are the most efficient constituents of the immune system that are capable of limiting tumor cell growth. Based on this bias, the goal of our studies

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has been to determine the best approach to induce strong and effective anti-tumor-specific T-cell responses as a means of developing epitope-based therapeutic vaccines for cancer.

T-Cells and Cancer

It is well accepted that the immune system has the ability to recognize and eliminate many types of tumors. As a consequence, significant efforts have been devoted in the last 20 years to the development of immune-based therapies for cancer. Cytotoxic T lymphocytes (CTL) and helper T lymphocytes (HTL) have been shown to react with antigens expressed by tumor cells and in many circumstances T-cell reactivity against tumor-derived antigens results in the induction of protective and therapeutic anti-tumor effects. While CTL can directly kill the tumor cells they recognize, antigen-specific HTL will amplify CTL responses and may also exhibit anti-tumor responses by producing lymphokines that directly inhibit tumor-cell growth. In several murine tumor model systems it has been clearly demonstrated that T-cells, and in particular CTL, are capable of eliminating established tumors. Adoptive transfer of tumor-reactive CTL,[1,2,5,6] active immunization using dendritic cells (DC) pulsed with CTL epitopes [7,8] or the use of strong co-stimulatory signals which increase CTL responses have all been reported as successful means of eliminating relatively large established tumors.

In humans, adoptive transfer of tumor-reactive T-cells (sometimes in combination with cytokines), has resulted in objective anti-tumor responses and in may cases in total tumor eradication.[5,6] Although these results have been most encouraging in limited types of tumors such as melanoma, renal cell carcinoma and B-cell lymphomas, positive responses are not observed in all patients. Furthermore, this mode of therapy has not been applicable to the most frequently encountered malignancies such as breast, lung, prostate and gastrointestinal carcinomas. With respect to active immunization, impressive therapeutic responses have been reported in melanoma patients immunized with peptides corresponding to CTL epitopes that were administered in combination with GM-CSF, IL-2 or pulsed onto DC.[11-14] However, as with adoptive therapy, not all patients responded favorably to this mode of immunotherapy and the applicability of this approach to other tumor types is limited because the appropriate CTL epitopes are yet to be defined.

The most likely explanation for these inconsistent results is that tumor cells vary significantly with respect to their antigenic composition and hence melanomas, renal carcinomas and B-cell lymphomas have been considered as "immuno-responsive", while most other tumors are regarded as "poorly immunogenic". Another possible cause for the variability observed in responses to T-cell adoptive therapy within the same tumor type is that the content of "antigen-specific" effector T-cells that are present in the cell product infused into the patients is usually not equivalent from patient to patient. Thus, the identification of relevant TAA and corresponding epitopes for tumor-reactive T-cells will certainly broaden the type of tumors suitable for immunotherapy and should increase the efficacy of therapeutic vaccines or adoptive therapy approaches by facilitating the induction of antigen-specific effector cells.

Although CTL are considered to be the main effector of anti-tumor immune responses, HTL play a pivotal role in enhancing tumor-reactive effector immune responses. Furthermore, HTL may also participate in the establishment of long-term immunity, which is essential for the prevention of tumor recurrences. It has been clearly demonstrated that restoration of long-term immunity by adoptive transfer of CTL requires the presence of antigen-specific HTL.[6,15,16] Thus, a T-cell mediated immunotherapy approach for tumors must include, in addition to the induction of CTL, the concurrent stimulation of TAA-specific T helper cells that will not only potentiate the therapeutic effect, but will also provide long-lasting immunological memory.

Antigen Recognition

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Antigen Recognition by T-Cells

T cells recognize antigen as small peptides bound to cell surface molecules encoded by the major histocompatibility gene complex (MHC). CTL are characterized by expression of CD8 cell surface molecules and by their capacity to induce lysis of the target cells they react with via the perforin/granzyme and/or the Fas/Fas-L pathways.[17,18] The T-cell receptors for antigen (TCR) of CTL bind to a molecular complex on the surface of the antigen-presenting cells (APC) formed by peptide epitopes usually derived from viral or tumor-associated antigens (TAA) and major histocompatibility gene complex (MHC) class I molecules. The peptides that are recognized by CTL are usually fragments 8-10 residues long that associate non-covalently with polymorphic class I MHC molecules.[19] On the other hand, HTL express the CD4 surface marker and recognize slightly larger peptides (12-20 residues) in the context of MHC class II molecules, which are only expressed in specific types APC such as B-lymphocytes, monocytes/macrophages and DC.[20]

Many normal and abnormal (e.g., oncogene products) cellular components as well as proteins derived from genes of foreign intracellular microorganisms are processed into MHC-binding peptides which are transported to the APC surface for presentation to the TCR.[19,20] After TCR engagement by appropriate MHC-peptide complexes, CTL have the ability to bind and kill target cells expressing foreign (infectious) or TAA. On the other hand, as a result of MHC-peptide recognition by HTL, these cells produce lymphokines that enhance and amplify CTL immune responses, or in some cases, HTL may also induce the lysis of the cell presenting the antigen or bystander cells via cytolytic mechanisms such as TNF and Fas ligand.

Strategies to Identify TAA and Selection of T-Cell Epitopes

Some of the changes that occur during cell transformation can produce MHC-binding peptides that are immunogenic for CTL or HTL. These TAA include: 1) oncogenic viral proteins; 2) abnormal overexpression of fetal or tissue specific proteins; and 3) mutated or overexpressed oncogene or tumor suppressor gene products.[21-26]

Over several decades various TAA such as CEA, PSA, HER2 and p53, which serve as "tumor markers" have been identified and biochemically characterized.[21,23-28] Because many of these antigens were first identified serologically or genetically, their relevance to CTL and HTL immunity was unclear. However, at the present time, there is sufficient evidence indicating that these tumor markers are capable of producing MHC-binding peptides that are recognized by CTL and HTL.

Two new approaches based on advanced molecular biology and immunology techniques has made it possible to identify several additional cellular products that can function as TAA for T lymphocytes. Both approaches rely on the availability of tumor-reactive CTL obtained from tumor bearing patients to screen gene libraries or peptide fraction isolates. T. Boon and collaborators pioneered the identification of TAA encoding genes of non-viral origin, originally in murine model systems and later in human melanomas.[22] This approach was used to identify a family of genes expressed predominantly in human melanomas (but also in a small proportion of breast, lung and colon carcinomas) and not in most normal tissues (with exception of the testes) designated MAGE.[29] Several CTL epitopes recognized by a melanoma patient's CTL were defined as 9 amino acid peptides which were presented to the TCR in association with class I MHC molecules. Various additional TAA (MART1/Melan-A; pmel-17/gp100, tyrosinase, gp75, p15, BAGE, GAGE, and others), also expressed mainly in melanomas, have been identified by several groups using the same methodology.[4,34-45] These proteins in addition to being expressed in melanomas, are also found in normal melanocytes. These observations demonstrate that under some circumstances TAA can be derived from normal cell constituents, and that immune tolerance to "self-antigens" at the CTL, and possibly at the HTL level is not necessarily always complete.[46] Another approach to identify TAA is to directly sequence

MHC-binding peptides that are eluted and purified from tumor cells.[47-49] This technique requires large numbers of tumor cells from which MHC molecules can be purified together with accurate and sensitive methods to characterize the eluted peptides and as in the previous method, TAA-reactive CTL, usually isolated from tumor patients are required to identify the active peptide fractions before they are sequenced by tandem-mass spectrography.

Identification of T-Cell Epitopes for Tumor Cells Using Reverse Immunology

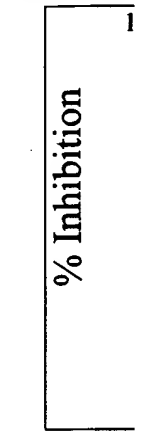
MHC-Binding Peptides as Potential T-Cell Epitopes

We have developed a completely different strategy to identify peptide epitopes for CTL which can be extended to HTL. These T-cell epitopes are derived from known TAA and the approach does not require the use of patient's tumor-reactive T-cells (which have been very difficult to isolate for tumors other than melanoma and renal-cell Ca) to screen DNA libraries or peptide fractions. This method involves three critical steps: i) identification of defined MHC binding motifs for the major HLA alleles; ii) selection of peptide sequences from putative or known TAA that contain these motifs and measurement of their capacity to bind to purified MHC molecules; and iii) determination of which MHC-binding peptides can elicit in vitro CTL that are capable of killing tumor cells that express the TAA. [50-53]

An important factor to consider in the identification of TAA is whether a peptide can bind to a specific MHC allele since MHC binding is a prerequisite for immunogenicity. Peptide binding to an individual MHC molecule depends on the specific sequence of the peptide.[19,54] Analysis of the sequence patterns of peptides that bind to MHC molecules in humans and mice has revealed the presence of primary and secondary anchor residues. MHC molecules are extremely polymorphic, and theoretically each allelic type will bind different sets of peptides (different alleles of the MHC tend to vary in those residues that form part of the peptide binding pockets). The MHC binding motifs for the most frequently found class I alleles (HLA-A1, -A2, -A3, -A11, -A24, -B7) as well as those for several major class II molecules (DRB*0101, -DRB*0301, DRB*0401 and DRB*0701) have been reported.[54-59] By identifying sets of tumor-associated peptides that bear these motifs and that bind to these the various HLA molecules, one could offer coverage to the majority of the human population (>80%) for developing T-cell epitope-based immunotherapy for tumors.

Once the selected TAA have been screened for sequences that contain MHC binding motifs, synthetic peptides representing these sequences can be synthesized and tested for their capacity to bind purified HLA molecules. Numerous quantitative peptide MHC binding assays have been developed which allow to screen a large number of motif-containing peptides from TAA and determine their binding affinity to several different HLA class I and II alleles. The results presented in Figure 1.1 illustrate an example of a quantitative binding assay of peptides derived from the MAGE antigens to HLA-A1 molecules. [51]

The last and probably most difficult step in the T-cell epitope identification process is to determine whether the peptides that have been identified as MHC binders are capable of inducing anti-tumor CTL or HTL responses. Primary T-cell immunization using synthetic peptides can be done in vitro using human peripheral blood mononuclear cells (PBMC) from appropriate HLA-typed individuals. In the following section we will present several examples where our group has been successful using this strategy in identifying numerous CTL epitopes for melanoma and solid epithelial tumors. The same approach should be applicable for the identification of tumor-reactive HTL.



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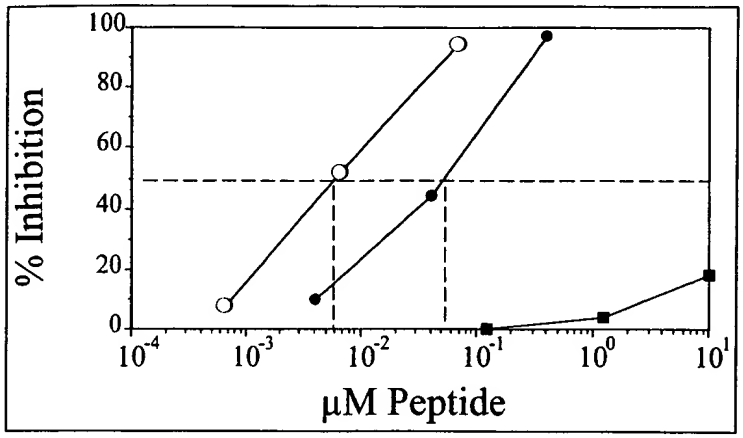


Fig. 1.1. HLA-A1 binding of synthetic peptides from MAGE-1, -2 and -3. The MAGE peptides were tested in a dose titration for the inhibition of the binding of the radiolabeled standard peptide ^{125}I-YLEPAIAKY to purified HLA-A1 molecules as described. Peptides tested were: (●), MAGE-1 peptide EADPTGHSY; (■), MAGE-2 peptide EVVPISHLY; (○), MAGE-3 peptide EVDPIGHLY. Dotted lines are used to calculate the 50% inhibitory concentration (IC_{50}) for each peptide, which inversely correlates with the binding affinity of the peptide to the HLA molecule. Reprinted with permission from: Celis E, Tsai V, Crimi C et al. Proc Natl Acad Sci (USA) 1994; 91:2105-2109. ©1994 National Academy of Sciences, USA.

In Vitro Immunization of T-Cells Using MHC-Binding Peptides from MAGE Antigens

The identification of MHC-binding peptides from known antigen sequences is not sufficient to guarantee that these peptides truly represent to T-cell epitopes. It is necessary to demonstrate that the T-cells can recognize cells that naturally process the antigen and present the corresponding peptide to the TCR as an MHC/peptide complex. In animals (mostly in mice) it is possible to immunize with peptides corresponding to putative T-cell epitopes and demonstrate that the responding T-cells can kill or proliferate to APC that process the corresponding antigen. In humans this type of approach is not feasible and one is limited to either evaluating the responses of T-cells isolated from patients or performing in vitro primary immunization of the T-cell precursors. As mentioned in previous sections, the use of patient-derived T-cell has limitations that in many types of cancer, especially the ones we wish to study here, antigen-specific T-cell lines/clones have been difficult to establish. Furthermore, as will be mentioned in more detail below, T-cells from patients will respond primarily to classical "immunodominant" epitopes whereas (in vitro) immunization studies using defined peptide epitopes should uncover both dominant and subdominant T-cell epitopes.[62]

In view of the above, we have developed an in vitro CTL immunization procedure that utilizes peptide-pulsed APC and CTL precursors from peripheral blood mononuclear cells (PBMC) of normal individuals.[61,63] Using this procedure our laboratory was the first to demonstrate the feasibility of inducing tumor-reactive CTL in normal individuals by in vitro immunization with peptide pulsed activated autologous epitopes B-cells as APC. The results presented in Figure 1.2 show that the HLA-A1-binding peptide that we identified from the MAGE-3 antigen (Fig. 1.1) was efficient in inducing peptide and tumor (melanoma) reactive CTL.

Subsequently, the technique of in vitro immunization of CTL was greatly improved by the use of tissue culture generated DC[64,65] that are used as professional APC. With DC we were able to generate primary CTL to a hepatitis B virus epitope in close to one 100% (1212)

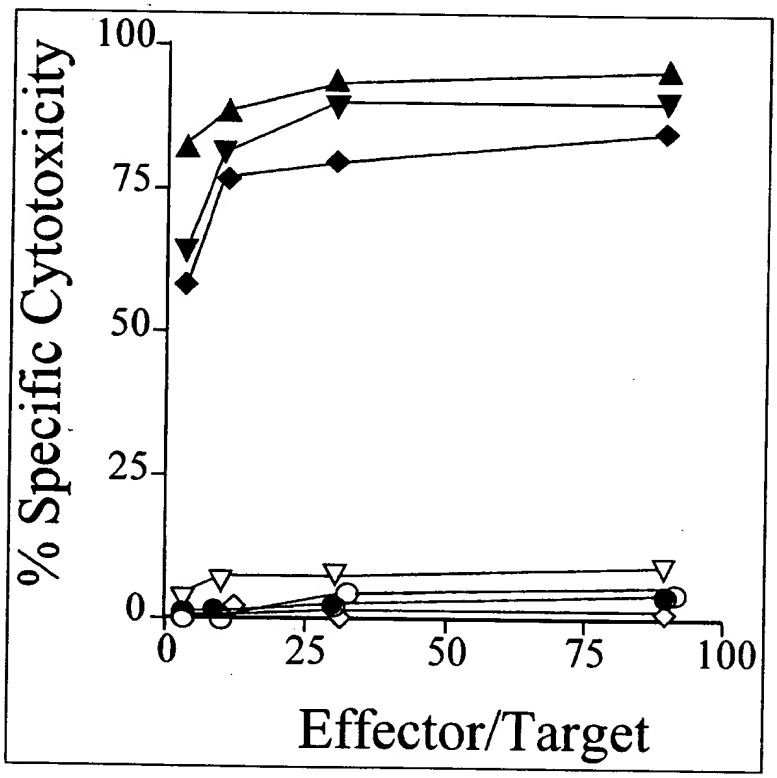
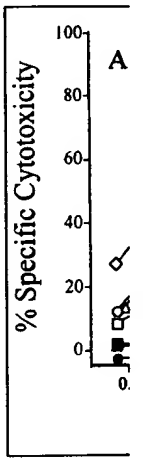


Fig.1.2. Antigen-specificity and MHC-restriction analysis of MAGE-3 reactive CTL. Cytotoxic responses using peptide-loaded target cells and melanoma tumors: (▲), Steinlin (HLA-A1$^+$ homozygous, Epstein-Barr Virus-transformed lymphoblastoid cell line (EBV-LCL), pulsed with MAGE-3 peptide EVDPIGHLY; (●), Steinlin cells (EBV-LCL) pulsed with MAGE-1 peptide EADPTGHSY; (○), Steinlin cells with no peptide; (▼), 397mel (HLA-A1$^+$, MAGE-3$^+$); (◆), 938mel (HLA-A1$^+$, MAGE-3$^+$); (◇), 888mel (HLA-A1$^+$ MAGE-3$^-$); (▽), 526mel (HLA-A2$^+$, MAGE-3$^+$). Reprinted with permission from: Celis E, Tsai V, Crimi C et al. Proc Natl Acad Sci (USA) 1994; 91:2105-2109. ©1994 National Academy Sciences, USA.

normal individuals. Moreover, the majority of the peptide-reactive CTL (>80%) were also capable of recognizing target cells that naturally process the hepatitis B virus epitope.[61]

Using the newly optimized DC immunization protocol, we proceeded to identify additional MAGE-specific CTL epitopes, but focusing on HLA-A2, one of the most frequently found MHC alleles in humans. In addition, we wished to determine whether MAGE-2 and MAGE-3-reactive CTL could recognize and kill other tumors besides melanomas, which may express these TAA. The rationale for this experiment is based on the published reports that approximately 20-60% of breast, colon and higher numbers of gastric carcinomas express the MAGE antigens.[66-68] Although numerous reports by several groups have demonstrated that MAGE-specific CTL were effective in killing MAGE$^+$ melanoma tumors,[30-33,52,69] no one had evaluated if these CTL could also recognize and kill MAGE$^+$ tumors of other type. Using peptide-pulsed DC, we tested the capacity of two HLA-A2-binding peptides from MAGE to induce tumor-reactive CTL by in vitro immunization of T-cells, and to determine whether these effector cells were capable of recognizing epithelial (non-melanoma) tumors expressing MAGE and HLA-A2 molecules. The results in Figure 1.3 clearly demonstrate that MAGE-2



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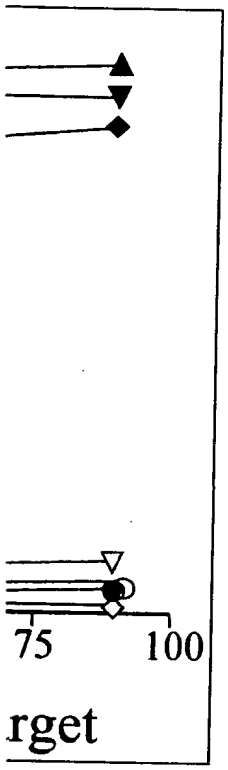

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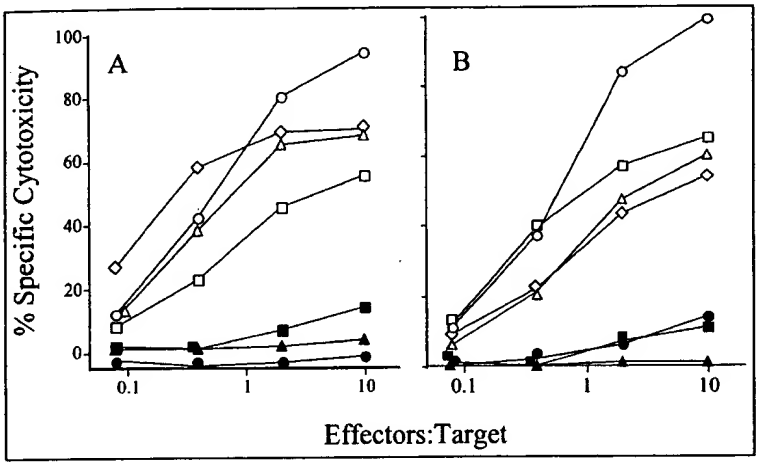

Fig. 1.3. Recognition of various tumor types by a MAGE-2 and -3 specific CTL clones. A MAGE-2 p157-166 (YLQLVFGIEV) specific CTL clone (A) and a MAGE-3 p112-120 (KVAELVHFL) specific CTL clone (B) were prepared from PBMC of a normal HLA-A2 donor using peptide-pulsed autologous DC as APC. Both clones were tested for their lytic activity against the following target cells: ○, .221A2.1 (HLA-A2* homozygous, EBV-LCL) pulsed with the corresponding MAGE peptide; ●, .221A2.1 cells without peptide; △, 624mel (melanoma, HLA-A2*, MAGE-2*/3*); □, KATO-III (gastric Ca, HLA-A2*, MAGE-2*/-3*); ◇, SW403 (colon Ca, HLA-A2*, MAGE-2*/3*); ■, WiDr (colon Ca, HLA-A2-, MAGE-2-/-3-); ▲, 888mel (melanoma, HLA-A2-, MAGE-2*/-3*). Reprinted with permission from: Kawashima I, Hudson S, Tsai V et al. Human Immunol 1998; 59:1-14. ©American Society for Histocompatibility and Immunogenetics, 1998 Elsevier Science, Inc.

and MAGE-3 specific CTL that were induced in vitro with peptide-pulsed DC, were very efficient in killing MAGE* melanoma, colon and gastric tumor cell lines.[70]

In collaboration with scientists from Takara Biotechnology, (Japan) we have identified additional new MAGE-specific CTL epitopes that are restricted by HLA-A24 which is the most common MHC class I allele in the Japanese population. The CTL elicited by the HLA-A24-binding peptides were efficient in killing both melanomas and gastric carcinomas that express the MAGE antigens.[72] These results confirm the prediction that immunotherapy using MAGE antigens may be applicable to tumors other than melanoma. However, because of the relatively low frequency of epithelial tumors that express MAGE, the use of additional TAA must be considered to offer adequate disease coverage.

In Vitro Immunization of Tumor-Reactive CTL Using MHC-Binding Peptides from Epithelial Tumor Markers

Epithelial sold tumors such as lung, gastrointestinal, breast and prostate represent the most common type of malignancies in the human population. Unfortunately, at present there is no knowledge of a single TAA that is expressed in the majority of these types of tumors which could be developed into a therapeutic vaccine. In view of this, it will become necessary to utilize several TAA to provide disease coverage for any immune-based therapeutic approach to treat epithelial-derived tumors. Our laboratory has selected in addition to MAGE, two TAA, HER2 and CEA, both which are found in a significant number of epithelial tumors. In the following we describe our efforts to identify CTL epitopes for these TAA.

A significant proportion (30-60%) of transformed breast epithelial cells overexpress and produce a proto-oncogene product known as HER2 (also known as neu, c-ErbB2, or $p185^{HER2}$),

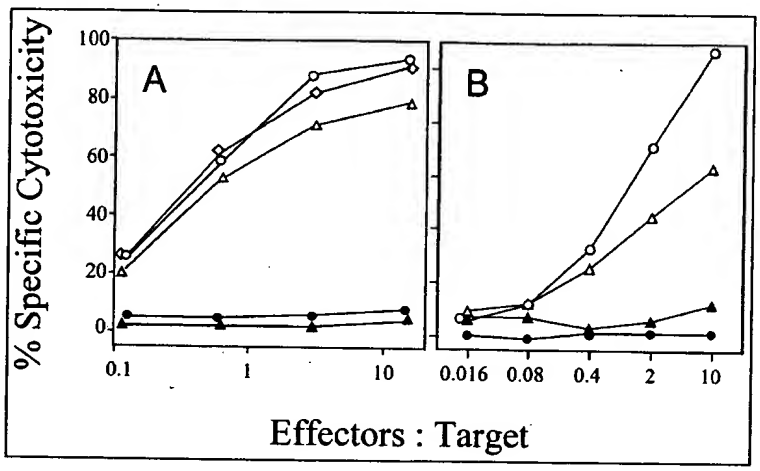

Fig. 1. 4. Cytotoxic T lymphocytes induced with HLA-binding peptides from HER2 can kill tumor cells. CTL were induced with the HLA-A2-binding peptide, HER2 p435-443 (ILHNGAYSL) (**panel A**) or the HLA-A3 binding peptide HER p754-763 (VLRENTSPK) (**panel B**). Peptide specific CTL were used as effector cells to test for the lysis of following target cell lines: ○, EBV-LCL cells of the appropriate HLA type pulsed with HER p435-443 (in A) or HER p754-763 (in B); ●, EBV-LCL cells without peptide; △, SW403 cells (colon Ca, HLA-A2$^+$/-A3$^+$, HER2$^+$); ▲, colon Ca cell line, A2$^-$/A3$^-$, HER2$^+$(negative controls). Reprinted with permission from: (A) Kawashima I, Hudson S, Tsai V et al. Human Immunol 1998; 59:1-14. ©American Society for Histocompatibility and Immunogenetics, 1998 Elsevier Science, Inc. (B) Kawashima I, Tsai V, Southwood S et al. Cancer Res 1999; 59:431-435. ©1999 Amer Assoc Cancer Res, Inc.

which bears some homology to the epidermal growth factor receptor.[73] Furthermore, the amplification and overexpression of this proto-oncogene on breast tumors has been associated with aggressive disease and poor prognosis. Other tumors, mainly adenocarcinomas of the ovary, colon and lung have also been reported to overexpress HER2. Since HER2 is selectively overexpressed in malignant cells and not in normal tissues, it has been considered as a possible antigen for CTL-mediated immunotherapy.[23,75,76] Indeed, there are several reports demonstrating that some CTL from ovarian cancer patients are capable of recognizing peptides derived from the HER2 protein.[23,25,77-79] Furthermore, there is also some evidence that helper T cells are capable of reacting with MHC class II peptides from HER2.[76,80,81]

Using our T-cell epitope identification strategy we tested the capacity of MHC class I-binding peptides from HER2 to induce tumor-reactive CTL. Several HLA-A2 and a few HLA-A3 binding peptides from HER2 were analyzed for their ability to trigger primary CTL responses using DC as APC. The examples presented in Figure 1.4 demonstrate that CTL possessing a high level of cytotoxicity for HER2+ tumor cells were produced to both HLA-A2 and HLA-A3 MHC molecules.[70,82]

Another TAA which is an ideal candidate for immunotherapy is CEA (carcinoembryonic antigen). CEA is a 180-kD glycoprotein that is extensively expressed on the vast majority of colorectal, gastric, and pancreatic carcinomas, it is also found in approximately 50% of breast cancers and on 70% of non-small-cell lung cancers.[28,83-87] CEA is also present, but at usually at lower concentrations, in the normal colon epithelium and in some fetal tissues. Circulating CEA can be detected in the great majority of patients with CEA positive tumors and has been used to monitor responses to therapy and disease progression. Recently, CEA-reactive CTL

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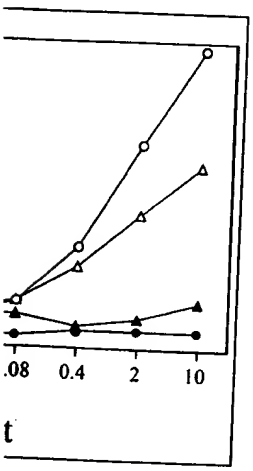

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responses were reported in cancer patients that were immunized with a recombinant vaccinia virus expressing CEA.[88] Following the same strategy as described above, we have identified several novel CTL epitopes restricted by commonly found MHC class I alleles such as HLA-A2, -A3 and -A24.[70,82,89] The results presented in Figure 1.5 show that as with other TAA, CEA-reactive CTL that are induced with MHC-binding peptides exhibit significant lytic activity towards CEA-expressing tumor cells.

Enhancement of T-Cell Immunogenicity by Epitope Manipulation

The main consideration of using individual T-cell epitopes instead of whole proteins for developing immune-based therapy for cancer is that while vaccinations using intact proteins representing "self antigens" rarely induce CTL and T helper responses, peptides when administered correctly, have been shown to be capable of eliciting strong T-cell responses to tumor cells.[80] Another major advantage of identifying and utilizing tumor-associated T-cell epitopes is the possibility of increasing their biological activity by the substitution of critical residues which are predicted to enhance MHC binding and/or TCR reactivity. There are several reports demonstrating that peptide analogues of CTL and HTL epitopes can be 10-1000X more efficient in stimulating antigen-specific T cells than the natural peptide sequences. For example, it was clearly shown that peptide analogues corresponding to two well known HLA-A2-restricted CTL epitopes from the melanoma-associated antigens gp100/pmel-17, p280-288 (YLEPGPVTA) and from MART-1/Melan-A, p26-35 (EAAGIGILTV), were shown to be significantly more potent in triggering CTL responses in vitro.[90,91] The analogs YLEPGPVTV and ELAGIGILTV contained substitutions in the primary HLA-A2 binding anchors (shown underlined), which increased the binding of the peptide to this MHC molecule.

In addition to the above examples, our group has demonstrated that a non-immunogenic peptide from CEA can be engineered to elicit tumor-reactive CTL in vitro.[70] In these studies we wished to address whether a low/intermediate HLA-A2-binding peptide that was not capable of inducing CTL could be rendered immunogenic by modifications designed to enhance its MHC binding. Specifically, peptide CEA p24-33 (LLTFWNPPT) is missing one of the "canonical" A2.1 anchors (a V at the carboxyl terminal end), as defined by pool sequencing analysis.[54] In order to determine whether substituting the non-canonical C-terminal anchor (T) with the canonical residue, a peptide analog from CEA p24-33 was prepared and tested for binding to purified HLA-A2.1 molecules. This analog had a "V" at the carboxyl-terminal end and an "M" at position 2 (CEA p24-33/M2V9, sequence: LMTFWNPPV), based on the finding that the presence of "M" in position 2 is frequently associated with optimal HLA-A2.1 binding capacity.[55,92] The analog peptide bound to purified HLA-A2.1 molecules with an approximately 40-fold increase CEA p24-33/M2V9 (IC_{50} 4.5 nM), as compared to the natural peptide, CEA p24-33 (IC_{50} 178.6 nM). It was notable that although peptide CEA p24-33 was not capable, at least according to our experimental protocol, of triggering CTL responses in vitro (data not shown), the peptide analog CEA p24-33/M2V9 was immunogenic in terms of CTL induction. A CTL clone induced by the analog specifically recognized and killed colon and gastric cancer cells expressing the CEA and HLA-A2 antigens (Fig. 1.6).

It has been reported that MHC class II HTL epitopes can also be engineered to increase their MHC binding affinity and T-cell immunogenicity. For example, a melanoma-reactive T helper line specific for the tyrosinase antigen and restricted by HLA-DRB*0401 was shown to recognize a peptide analog with a substitution in one of the major MHC anchors, 10-100 X better than the natural peptide sequence.[93]

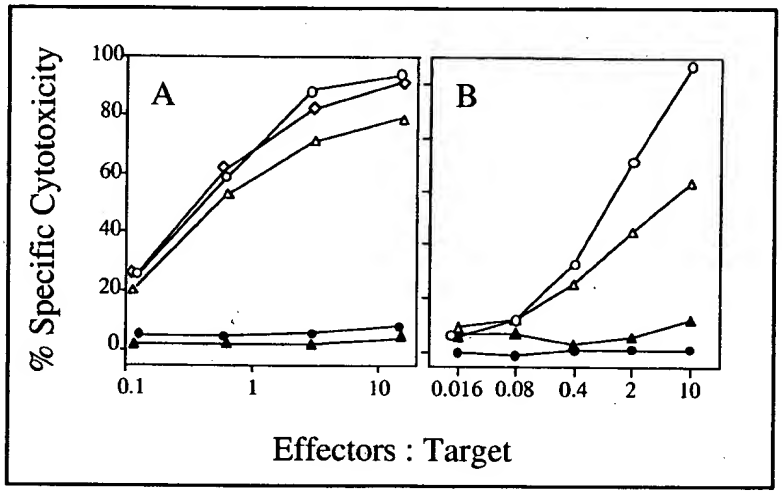


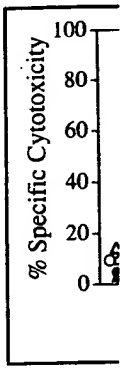
Fig. 1.5. Cytotoxic T lymphocytes induced with HLA-binding peptides from CEA can kill tumor cells. CTL were induced with the HLA-A2-binding peptide, CEA p691-700 (IMIGVLVGV) (panel A) or the HLA-A3 binding peptide CEA p61-70 (HLFGYSWYK) (panel B). Peptide specific CTL were used as effector cells to test for the lysis of following target cell lines: O, EBV-LCL of the appropriate HLA type pulsed with CEA p691-700 (in A) or CEA p61-70 (in B); ●, EBV-LCL without peptide; △, SW403 cells (colon Ca, HLA-A2$^+$/-A3$^+$, CEA$^+$); ◇, KATO-III cells (gastric Ca, HLA-A2$^+$, CEA$^+$); ▲, colon Ca cell line, HLA-A2$^-$/-A3$^-$, CEA$^+$(negative controls). Reprinted with permission from: (A) Kawashima I, Hudson S, Tsai V et al. Human Immunol 1998; 59:1-14. ©American Society for Histocompatibility and Immunogenetics, 1998 Elsevier Science, Inc. (B) Kawashima I, Tsai V, Southwood S et al. Cancer Res 1999; 59:431-435. ©1999 Amer Assoc Cancer Res, Inc.

In summary, it is clear that T-cell epitope identification allows the unique advantage of designing more potent immunogens based on the modification of peptide sequences aimed towards increasing MHC binding affinity. Needless to say, this approach can be extremely useful when the targeted antigen represents a molecule which may be expressed in normal cells such as most TAA, where immune tolerance may need to be broken.

Conclusions

Therapeutic Approaches Using Defined T-Cell Epitopes

The ultimate goal of our research is to develop effective immunotherapies for both early (pre-metastatic) and advanced cancer patients utilizing the information derived from our T-cell epitope identification and re-engineering efforts. Because most patients suffering with advanced, metastatic disease are likely to be immunosuppressed due to their overall poor health status or as a result of chemo/radiation therapy, the logical approach to carry out anti-tumor immune intervention would be adoptive cellular therapy. TAA-specific CTL and HTL could be prepared in vitro as described above using CD8$^+$ and CD4$^+$ T-cells stimulated with autologous peptide-pulsed DC. It is foreseeable that in tissue culture (ex vivo) most of the immunosuppressive factors affecting the cancer patient can be eliminated. The in vitro-generated antigen-specific T-cells will then be expanded either by sequential stimulation with antigen or with mitogen (e.g., anti-CD3 antibody) as described by Riddell and Greenberg and later re-infused into the patients with or without the addition of systemic lymphokines (e.g., IL-2).



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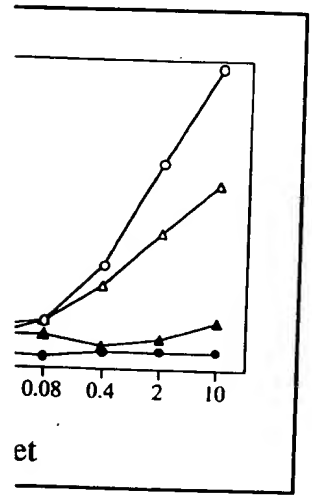

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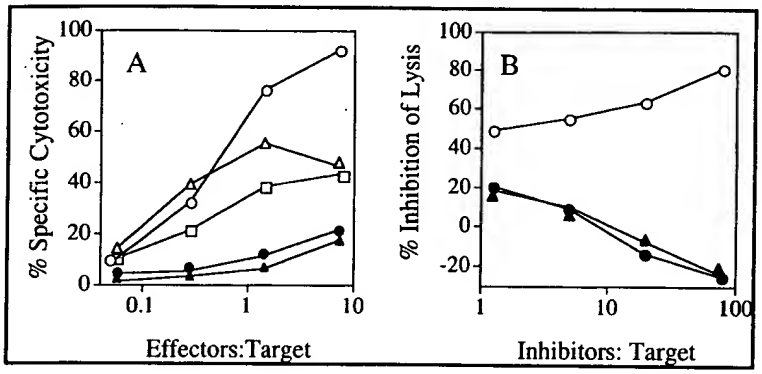

Fig. 1.6. Peptide analogs can induce tumor-reactive CTL. **Panel A:** The CEA p24-33/M2V9 (analog, sequence: LMTFWNPPV) specific CTL clone was tested for its cytolytic activity against following target cell lines: ○, EBV-LCL cells pulsed with CEA p24-33/M2V9; ●, EBV-LCL without peptide; △, SW403 (colon Ca, HLA-A2⁺, CEA⁺); □, KATO-III (gastric Ca, HLA-A2⁺, CEA⁺); ▲, HT-29 (colon Ca, HLA-A2⁻, CEA⁺). **Panel B:** Antigen specificity demonstrated by cold target inhibition assay. Lysis of ^{51}Cr-labeled SW403 cells at an effector/target ratio of 2:1 by the CEA p24-33/M2V9 specific CTL clone was blocked at various Inhibitors / Target ratios by the following cold targets: ○, EBV-LCL pulsed with CEA p24-33/M2V9; ▲, EBV-LCL pulsed with irrelevant HLA-A2.1 binding peptide (FLPSDYFPSV); ●, EBV-LCL without peptide. Reprinted with permission from: Kawashima I, Hudson S, Tsai V et al. Human Immunol 1998; 59:1-14. ©American Society for Histocompatibility and Immunogenetics, 1998 Elsevier Science, Inc.

Anti-tumor active immunotherapy (i.e., vaccination), on the other hand, will be more suitable for early (non-metastatic) disease, where patients are most likely to have an intact immune system. Furthermore, it becomes very attractive to utilize anti-tumor vaccines in the adjuvant setting with the aim of preventing tumor recurrences after primary conventional treatment by the elimination of micrometastases. Several potential approaches for inducing effective in vivo CTL and HTL responses to TAA can be contemplated. In many circumstances, vaccination with synthetic peptides representing the defined CTL and HTL epitopes in the absence or presence of adjuvants appears to be an easy and attractive way of inducing T-cell responses.[94-96] However, although this mode of vaccination can effectively prevent the establishment of tumors (prophylaxis) it seldom has any benefit when used in the therapeutic mode. Furthermore, it has recently been reported that vaccination with certain peptides representing CTL epitopes can result in T-cell inactivation/deletion[97,98] which would certainly be an undesirable outcome for the treatment of tumors. Notwithstanding the above concerns, it was recently reported that some melanoma patients exhibited tumor responses after receiving peptide vaccination in combination with GM-CSF or IL-2.[11-13] These results support further research in this area, where optimization of vaccination protocols could become an effective method of anti-tumor immunotherapy.

Another approach for inducing active immunity against TAA, especially when multiple T-cell epitopes are being considered, is the use of DNA-based vaccines. Plasmid vaccines containing multiple T-cell epitopes linked in tandem have been reported to induce strong T-cell responses to most of the components of the vaccine. Nevertheless, it remains to be determined whether these types of immunogens are capable of inducing sufficiently potent immune responses capable of providing benefit (extend disease-free survival) in cancer patients.

Currently, the most promising type of anti-tumor vaccine is the use of autologous peptide-pulsed DC. As mentioned previously, DC can relatively easily be prepared in tissue culture from monocytic precursors ($CD14^+$ cells) that are incubated for approximately 1 week with GM-CSF and IL-4. Experiments in mouse models have shown that peptide-pulsed DC vaccination can effectively eliminate established tumors and extend disease-free survival.[7,8] Also very encouraging is the recent observation that several melanoma patients vaccinated with autologous DC pulsed with melanoma-associated peptides demonstrated objective tumor responses.[14] Thus, it appears that antigen-presenting DC are capable of overcoming potential immune tolerance and triggering immune responses to epitopes that are expressed in some normal tissues (melanocytes).

Finally, regardless of the vaccination strategy selected to induce anti-tumor CTL and HTL responses in individuals with established tumors, it becomes critical to carry out clinical studies with realistic endpoints. Unfortunately the evaluation of anti-tumor immunotherapy has been set to the standards of conventional cancer treatments such as chemo and radiation therapy where effectiveness is the reduction or elimination of measurable tumors. Many tumor immunologists, including myself and Dr. Martin Kast feel quite strongly that is naive to expect the disappearance of large tumor masses as a result of vaccination. More realistic and desirable endpoints such as 1) disease free survival, 2) overall survival with reasonable quality of life and 3) time to recurrence should be used to evaluate immunotherapy in cancer. Unfortunately, clinical studies designed to measure these endpoint require significant number of patients, a considerable amount of time (2-5 years minimal), a high amount of resources, and most importantly patience on the part of the clinical investigators and for-profit companies involved in the testing of potential new compounds.

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CHAPTER 2

Mutant Onc Gene Produ by Chromos for Cancer V

V. Ell n Maher, B. S Michael J. Kelley, M Leon van den Broek and Jay A. Berzofsk

Introducti n

Active immunoth is strong evidenc and levamisole, been used as therapies system include tumors withdrawal of that thera examples of the efficacy a clinical response in pat has grown with our unc schemes have been carri these trials have been ch the specificity of tumor sen, is to use our increas Molecular changes whi mutant proteins and ch a unique immunologic of overexpressed and m ated by chromosomal t tional advantage that t cell, so that escape mu malignant phenotype.

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